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Characterization of the highly branched glycogen from the thermoacidophilic red microalga *Galdieria sulphuraria* and comparison with other glycogens

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ABSTRACT

The thermoacidophilic red microalga *Galdieria sulphuraria* synthesizes glycogen when growing under heterotrophic conditions. Structural characterization revealed that *G. sulphuraria* glycogen is the most highly branched glycogen described to date, with 18% of α -(1 $\rightarrow$ 6) linkages. Moreover, it differs from other glycogens because it is composed of short chains only and has a substantially smaller molecular weight and particle size. The physiological role of this highly branched glycogen in *G. sulphuraria* is discussed.

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1. Introduction

Energy storage molecules are important in the physiology of cells during periods of transient nutrient starvation often encountered in nature. Starch (present in plants and various species of green and red algae) and glycogen (present in some bacteria, yeast and animal cells) are two of the most common energy reservoirs molecules. Both are glucose polymers with a linear backbone of α -(1 $\rightarrow$ 4) linked units and branches attached at certain points through α -(1 $\rightarrow$ 6) linkages. However, they differ in the percentage and distribution of branches, which at the end influences the structure and properties of the molecule itself [1]. Starch is composed of two types of polymers: amylose, with virtually no branching points, and amylopectin, with 5–6% of α -(1 $\rightarrow$ 6) linkages distributed in a non-random manner [2]. The branched regions are arranged in clusters separated by linear regions, leading to the appearance of crystalline sections and the formation of insoluble starch granules [3]. Glycogen contains shorter linear chains compared to amylopectin and a higher number of random branching points arranged

in tiers [4]. The consequence is that glycogen adopts a globular form which is highly soluble in water because it prevents chain interaction and granule formation. The branching degree of glycogen depends on its source and ranges from 7 to 13% [5–9].

Red algae (Rhodophyta) are a group of photosynthetic eukaryotes that include unicellular and multicellular species. The energy storage compound of red algae is generally called floridean starch, named after one of the taxonomic groups in which Rhodophyta is classified [10]. Earlier research was focused on describing the differences in the precursor molecule and the intracellular location of the floridean starch pathway with respect to the plant and green algae starch [11]. However, more recent studies have shown that the composition of floridean starch, regarding the presence or absence of amylose [12], and the structure, regarding the level of branching, varies among species [13–15]. *Galdieria sulphuraria* is an unicellular red alga that is considered to be one of the most primitive eukaryotes on earth [16]. It is able to thrive in acidic environments with pH values from 0 to 4 at temperatures up to 56 °C, conditions found in hot sulfur springs and volcanic areas around the world. The harsh conditions in which this alga lives have pushed *G. sulphuraria* towards adaptation through the development of metabolic flexibility [17], being able to grow autotrophically, performing photosynthesis when light is available, and heterotrophically, using

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several organic compounds as carbon source in the absence of light [18]. The glucan accumulated by *G. sulphuraria* as energy reservoir has been analyzed in a previous work whose aim was to point out the differences in the structure of this molecule between several red algae species belonging to the same order [13]. This study showed that a strain of *G. sulphuraria* isolated from Japan and grown autotrophically in a concentrated mineral medium accumulated a glycogen-type of molecule, differing from the other members of its order, in which a plant-like starch or semi-amylopectin type of molecule was synthesized.

In this study, the structure of glycogen extracted from the red microalga *G. sulphuraria* strain SAG 108.79 native to Yellowstone National Park growing under heterotrophic conditions is characterized in detail and compared to prokaryotic and eukaryotic glycogens using the same experimental procedures.

2. Materials and methods

2.1. Materials

Glycogen from Oyster type XI was purchased from Sigma-Aldrich. Glycogens from *Pseudomonas* V-19, *Sphaerotilus natans* and *Arthrobacter viscosus* were a kind gift from dr. M. Breedveld (Groningen), who obtained the samples from late dr. L. Zevenhuizen (Wageningen University).

2.2. Strains, media and growth conditions

G. sulphuraria strain SAG 108.79 was obtained from the culture collection of the University of Göttingen (Sammlung von Algenkulturen, Germany). Cells were maintained growing on 1.5% agar plates of Allen's mineral medium [19] at pH 4 and 40 °C with constant illumination of 100 $\mu\text{E}/\text{m}^2\text{s}^{-1}$. Colonies were transferred to a fresh plate once a month. For glycogen extraction, *G. sulphuraria* cells were grown in 1 L flasks containing 500 mL of Allen medium at pH 2 supplemented with 1% (w/v) glycerol at 40 °C in darkness on a rotary shaker at 150 rpm.

Escherichia coli strain DSM 10235 and *Arthrobacter globiformis* strain DSM 20124 were purchased from the culture collection of the Leibniz Institute (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany). For glycogen extraction, *E. coli* and *A. globiformis* cells were grown in 1 L flasks containing 200 mL of M9 mineral medium at pH7 supplemented with 0.5% (w/v) tryptone (BD Bacto) and 1% D-glucose at 37 °C (for *E. coli*) or 30 °C (for *A. globiformis*) on a rotary shaker at 180 rpm.

2.3. Glycogen extraction from *G. sulphuraria*

G. sulphuraria cells were grown until late exponential phase and harvested by centrifugation at 10,000 $\times g$ for 5 min at room temperature. The cell pellet was washed twice with ultra-pure water and freeze-dried. Dry cells were resuspended in 20 mL of ultra-pure water, mixed with 10 mL of glass beads (425–600 μm , Sigma-Aldrich) and disrupted by shaking for 10 min in a Mixer Mill (MM400, Retsch) at a frequency of 30 Hz. The cell lysate was transferred to a new tube and centrifuged at 20,000g for 10 min. The supernatant, free of cell-debris and unbroken cells, was incubated in a waterbath at 100 °C for 15 min to denature and precipitate proteins and then centrifuged at 20,000g for 20 min. The supernatant was transferred to a new tube and 0.1 volumes of 50% trichloroacetic acid (TCA) were added to further precipitate residual proteins. The sample was incubated on ice for 10 min and then centrifuged at 20,000g for 20 min. The clear supernatant was mixed with 1 volume of absolute ethanol to precipitate the glycogen at 4 °C. Precipitated glycogen was recovered by centrifugation at

10,000g for 10 min and freeze-dried. The dry glycogen was resuspended again in ultra-pure water, precipitated with 1 volume of absolute ethanol and freeze-dried.

To exclude a possible influence of intracellular amylase activity during extraction on the final structure of the glycogen, two additional versions of the extraction protocol were also tested. In one of the versions, cell disruption was performed at low temperature and the sample was always kept on ice in between steps. The dry cells were resuspended in cold (4 °C) ultra-pure water and disrupted by bead beating in pre-cooled (–20 °C) jars containing glass beads. The cell lysate was centrifuged at 20,000g for 10 min at 4 °C to remove cell debris and the supernatant was then boiled at 100 °C for 15 min to denature and precipitate proteins. From this point on, the sample was further treated as described above. In the other version, intracellular enzymes were inactivated prior to cell disruption by boiling the cell suspension at 100 °C for 15 min. Cell disruption and glycogen extraction was continued as described above.

2.4. Glycogen extraction from *E. coli* and *A. globiformis*

E. coli and *A. globiformis* were grown on M9 media containing tryptone and glucose until late exponential phase. Cells were harvested, washed with sterile water, resuspended in the same volume of M9 media containing glucose but not tryptone and incubated under the same conditions for the same amount of time as before. Then, cells were harvested by centrifugation at 20,000g for 10 min at 4 °C, the cell pellet washed twice with ultra-pure water and freeze-dried. The dry pellet was resuspended in ultra-pure water and cells were disrupted by passing them 5 times through an EmulsiFlex B-15 disruptor (Avestin) at a homogenizing pressure of 36,000 psi, cooling down on ice between disruptions. Cell debris and unbroken cells were removed by centrifugation at 20,000g for 10 min at 4 °C and the cell lysate was further treated in the same way as described in Section 2.2 to precipitate the glycogen, with the exception that no boiling step was performed.

2.5. ^1H NMR analysis

5 mg of glycogen was dissolved in 600 μL of deuterium oxide (D_2O , 99.9% $_{\text{atom}}$, Sigma-Aldrich), freeze-dried and exchanged in the same solvent two more times. Samples were measured in a NMR 500 Hz spectrometer (Varian) with the probe temperature set at 80 °C to avoid overlapping of the peaks of interest with the signal originating from residual water in the sample, thus allowing a more precise integration. For an accurate quantification of the branched and linear linkages in the molecule based on the integration of the signals, special attention was paid to the delay time between pulses in order to allow all protons to fully relax before a new pulse was applied. An experiment to measure the T1 (spin-lattice) relaxation times of the protons of interest was carried out following the inversion-recovery method with 10 tau values. The longest T1 (that of the H1 in the α -(1 $\rightarrow$ 6) linkage) was 1.49 s and, in consequence, the relaxation delay during spectra acquisition was set to 25 s, thus more than 5 times the longest T1 value, as recommended in literature [20]. The spectra were acquired by running 16 scans with a 90° pulse. Acetone ($\delta^1\text{H}$ 2.225 ppm in D_2O) was used as internal reference for chemical shift assignment. Data were analyzed using MestReNova 9.1.0 (Mestrelab Research S.L.). The percentage of each type of linkage was calculated setting the total integral value of both peaks to 100.

2.6. Chain length distribution

Glycogen samples were dissolved in ultra-pure water to a final concentration of 20 mg/mL and then 500 μL of this solution were mixed with 3 volumes of sodium acetate buffer 200 mM pH 4.5 con-

Fig. 1. ^1H NMR spectra of the glycogen from *G. sulphuraria* in D_2O at 80°C . The signal originated by the residual water in the sample (HOD peak at 4.24 ppm) was cut off from the spectrum.

taining 5 mM of CaCl_2 . The samples were debranched by adding 1 unit of Isoamylase from *Pseudomonas* sp. (Megazyme) and 1.8 units of Pullulanase M2 from *Bacillus licheniformis* (Megazyme) and incubating at 40°C for 48 h with constant mixing by shaking. The enzymes were inactivated by boiling at 100°C for 5 min and then centrifuging at 10,000g 5 min. Samples were analyzed by high pH anion exchange chromatography coupled with pulse amperometric detection (HPAEC-PAD) on a DX 500 workstation equipped with a CarboPac PA-1 column and an ED40 detector (Dionex), using a linear gradient of 0–300 mM sodium acetate in 100 mM of NaOH in 30 min. All glycogen samples were analyzed on the same system using the same settings. A mixture containing oligosaccharides from glucose to maltoheptaose was used as a standard for peak assignment. The amount of each chain was calculated as a percentage relative to the total peak area.

2.7. Molecular size distribution

Glycogen samples were prepared at a concentration of 2 mg/mL in dimethyl sulfoxide (DMSO) containing 0.05 M of lithium bromide and filtered through a $0.2\ \mu\text{m}$ membrane. The samples were analyzed by size exclusion chromatography (SEC) on an Agilent Technologies 1260 Infinity system (PSS) equipped with a PFG guard-column and three PFG SEC-columns 100, 300 and 4000 (PSS), using DMSO/0.05 M LiBr as eluent at a flow rate of 0.5 mL/min. The data from the multiangle light scattering (SLD 7000, PSS) and refractive index (G1362A 1260 RID, Agilent Technologies) detectors were processed with WinGPC Unity software (PSS). A $d\eta/dc$ value of 0.072 (that of pullulan in DMSO, private communication with PSS) was applied for molecular weight calculations [21].

2.8. Cryogenic transmission electron microscopy

Glycogen samples were prepared in ultra-pure water at a concentration of 1 mg/mL the same day of the experiment in order to prevent microbial growth. A few microliters of the solution were placed on a glow discharged holey carbon coat grid (Quantifoil 3.5/1),

blotted and vitrified in a Vitrobot (FEI) and observed in a Gatan model 626 cryo-stage on a Philips CM120 electron microscope operating at 120 kV. Images recorded on a slow-scan CCD camera under low-dose conditions.

3. Results

Glycogen was extracted from *G. sulphuraria* cells growing in complete darkness in a mineral medium at pH 2 supplemented with 1% (w/v) glycerol as the sole carbon source. Under these conditions, the amount of glycogen extracted was at least 20% of the dry cell weight (data not shown). Glycogen from *E. coli* and *A. globiformis* was extracted in our laboratory using the same protocol and was used for comparison in the analyses that were carried out, together with several prokaryotic glycogens and one commercially available glycogen from oyster.

To determine the proportion of linear and branched linkages, all glycogen samples were subjected to ^1H NMR. This technique represents a useful tool for the analysis of glucose polymers with α -(1 $\rightarrow$ 4) and α -(1 $\rightarrow$ 6) glycosidic bonds because the signals from the H1 (anomeric proton) in glucose residues involved in these linkages appear as two well isolated chemical shifts, downfield from the region of overlapping signals from the other protons in the glucose ring [22]. The ^1H NMR spectrum of the glycogen from *G. sulphuraria* (Fig. 1) showed a very pronounced peak at 4.98 ppm, originating from the H1 in (1 $\rightarrow$ 4)- α -Glucose-(1 $\rightarrow$ 6) residues, and a broader peak at 5.33–5.36 ppm, resulting from the two overlapping signals coming from the H1 in (1 $\rightarrow$ 4)- α -Glucose-(1 $\rightarrow$ 4) and (1 $\rightarrow$ 4,6)- α -Glucose-(1 $\rightarrow$ 4) residues. Integration of these two peaks in all glycogen samples showed that the glycogen of *G. sulphuraria* has the highest degree of branching of all, with 18% of α -(1 $\rightarrow$ 6) linkages (Table 1). This high proportion of branching linkages detected was not an artifact of the extraction protocol due to endogenous enzymes that were still active and might have degraded the linear chains of the molecule. Glycogen extracted from *G. sulphuraria* cells in which the potential activity of these enzymes was inhibited,

Fig. 2. Chain length distribution of some of the glycogens after debranching with isoamylase and pullulanase.

Table 1

Percentage of linear and branching linkages determined by ^1H NMR in the glycogens analyzed in this study.

Glycogen source	% α -1,6 linkages
<i>Galdieria sulphuraria</i>	18.0
<i>Pseudomonas V-19</i>	13.1
<i>Arthrobacter viscous</i>	13.5
<i>Arthrobacter globiformis</i>	11.1
<i>Sphaerotilus natans</i>	8.7
<i>Escherichia coli</i>	8.0
<i>Oyster</i>	7.1

either by low temperatures or by heat denaturation, showed no substantial differences in the % of α -(1 $\rightarrow$ 6) bonds (data not shown).

To further elucidate the structural properties of the different glycogens, they were subjected to a chain length distribution. The glucans were completely debranched by cleaving the α -(1 $\rightarrow$ 6) linkages with a combination of isoamylase and pullulanase and the resulting fragments were separated by HPAEC-PAD. The glycogen from *G. sulphuraria* showed a unique chain length distribution (Fig. 2), which differed markedly from that of the other glycogens in its lack of chains of DP > 10. The glycogens of *A. globiformis*, *A. viscous* and *S. natans* gave similar patterns as the *Pseudomonas* and oyster glycogens, respectively (data not shown). When plotting the percentage of short chains (DP $\leq$ 10) versus long chains (DP $\geq$ 15) (Fig. 3), the different glycogens could be divided into two groups. One group contained the glycogens where short branches represented around 60% or less of all chains, while the other included glycogens where the proportion of these branches was at least 80%,

Fig. 3. Comparison of the percentage of short chains versus long chains in the glycogens analyzed in this study.

with the notable example of *G. sulphuraria* glycogen, where virtually all chains (99.5%) are equal to or shorter than 10 glucose residues.

When subjecting the samples to SEC in order to describe the molecular size distribution, a clear difference could be observed. The glycogen of *G. sulphuraria* was composed of a population of smaller molecules with a weight-average molecular weight (M_w) of 2.5×10^5 Da (Fig. 4), which was clearly smaller than that of the other glycogens, all of them with a M_w of at least one order of magnitude bigger. In order to obtain information about the particle structure, the different glycogens were analyzed by cryogenic transmission electron microscopy (cryo-TEM). This technique allows the

Fig. 4. Molecular size distribution of some of the glycogens dissolved in DMSO/0.05 M LiBr.

Fig. 5. Cryo-TEM pictures of the glycogens at a concentration of 1 mg/mL. (A) *G. sulphuraria* (B) *Oyster* (C) *Pseudomonas V-19* (D) *S. natans*.

visualization of glycogen in suspension in a vitrified state, maintaining its hydration and native conformation and avoiding artifacts that might arise from sample drying during conventional TEM [23]. Fig. 5A shows that *G. sulphuraria* glycogen particles were significantly smaller than the other glycogens (Fig. 5B–D), which appeared as bigger β -particles and in some cases arranged as α -rosettes of up to 100 nm.

4. Discussion

The thermoacidophilic red microalga *G. sulphuraria* has been shown to differ from other red algae species of the same order because it synthesizes glycogen instead of semi-amylopectin as storage glucan [13]. The *G. sulphuraria* strain SAG 108.79 used in this study accumulates high amounts of glycogen when growing under heterotrophic conditions using glycerol as carbon source. The structure of this glycogen differs markedly from the structure of other glycogens from different sources. Firstly, *G. sulphuraria* glycogen has a much higher degree of branching (18%). To our knowledge, no naturally occurring glycogen (i.e. without being synthesized *in vitro* by the concerted action of recombinant expressed enzymes or by enzymatic modification of amylose or amylopectin) has been described to have a percentage of α -(1 $\rightarrow$ 6) linkages as high as 18%. This makes the glycogen of *G. sulphuraria* the most highly branched glycogen known to date. The related species *Galdieria maxima* has also been reported to synthesize a glycogen with a high degree of branching [24], albeit lower than the one reported here for *G. sulphuraria*. This suggests that the accumulation of a highly branched glycogen as storage molecule might be a distinctive trait of the members of the genus *Galdieria* and not restricted to a single species. The degree of branching obtained for the other glycogens used for comparison is in agreement with previous values reported in literature obtained by different methods [9], confirming once more that ^1H NMR is a valid technique for measuring the amount of branched linkages in glycogen provided that long enough relaxation times are used. As previously reported in literature [5–9], and confirmed once more in this study, there is not a general branching degree value for glycogen, but instead this number depends on the species from which the glucan is extracted. Secondly, the chain length distribution profile indicates that *G. sulphuraria* glycogen is almost entirely composed of short branches with $\text{DP} \leq 10$. Similar chain length distributions had been previously reported for other *G. sulphuraria* strains [13,14,25]. This implies that those chains of 10 glucose residues, being the longest in the molecule, are very likely bearing two branching points, if not more. In their work on evolutionary optimization of glycogen structure, Meléndez et al. [26] mention that the minimum distance between two branches is 4 glucose residues, since that is the physical space that the branching enzyme needs to carry out its activity. Therefore, a chain containing 2 or more branching points has to be necessarily formed by more than 10 glucose residues. Our data indicate that this is not the case for *G. sulphuraria* glycogen, suggesting that the branching enzyme of this microalga might have the ability to work with a more limited space and introduce α -(1 $\rightarrow$ 6) linkages in closer vicinity to other branching point than the branching enzymes from other microorganisms. Alternatively, the key to the structure of this highly branched glycogen could reside on the combined action of all the enzymes involved in glycogen synthesis/degradation in the cell. To answer this question it would be necessary to characterize the branching enzyme from *G. sulphuraria*. Finally, *G. sulphuraria* glycogen also differs from others in its substantially smaller M_w , as shown in the molecular size analysis, and particle size, observed by cryo-TEM. It has been pointed out in previous works that the factor which determines the final size of the individual glycogen particle (β -particle) is what is known as “crowding” mechanism

[27]: the glycogen particle grows during synthesis increasing its density exponentially with every new tier formed, until this density is so high that the synthesis cannot proceed further because the enzymes do not have enough space to carry out their activity. *G. sulphuraria* glycogen particles are significantly smaller than the others, which suggests that this density limit is reached earlier during synthesis due to the higher percentage of branching linkages. Moreover, α -particles could not be observed for *G. sulphuraria* glycogen. The mechanism by which individual β -particles group together forming bigger α -particles is not completely unraveled but it seems to differ with the glycogen type. Powell et al. [28] proposed a “crowding/budding” mechanism involving glycosidic linkages for the formation of α -particles in phytoglycogen, in which synthesis continues from a branch that escapes the outer layer of the glycogen particle until a new β -particle arises. In the case of *G. sulphuraria* glycogen, the outer layer of the particle might be too crowded and the chains too short to escape it, impeding the glycogen synthase from continuing its activity and therefore α -particle formation.

From a physiological point of view, the question is why *G. sulphuraria* accumulates such a highly branched glucan. One possibility is that this feature enhances its role as a short-term energy storage molecule. Glycogen has always been considered an easily accessible energy reservoir due to its branched nature, which increases the number of residues that are exposed to the attack of enzymes responsible for its degradation. Thus, the higher the percentage of branches a glycogen molecule contains, the more rapidly it can be degraded to provide the cells with energy. This seems plausible if we consider that *G. sulphuraria* can colonize hostile habitats and has evolved to be metabolically versatile and resistant to different stresses [17], a survival strategy that demands from the cells a constant supply of energy to be used for adaptation. However, Wang and Wise [9] formulated a complete opposite theory, suggesting that glycogens with shorter average chain length, which is directly related to a higher degree of branching, function as long-term energy storage molecules because α -(1 $\rightarrow$ 6) linkages are degraded by enzymes at a lower rate than α -(1 $\rightarrow$ 4) linkages. In fact, it has been described that *G. sulphuraria* is able to survive long periods in complete darkness [29]. Thus, a highly branched glycogen that is degraded at a low rate would enable the cells to keep a supply of energy over a long period of time to maintain their metabolism and ensure their survival. *G. sulphuraria* is also known to be tolerant to high osmotic concentrations. This alga lives on the surface, or even in internal layers of rocks [30] in environments with relatively high temperatures, where evaporation of water often leads to an increase in the concentration of solutes outside the cells. Therefore, cells need to compensate the extracellular osmotic pressure by accumulating solutes in their cytoplasm that will prevent the loss of intracellular water. Since *G. sulphuraria*, like the rest of red algae, accumulates its storage glucan in the cytosol and not in its chloroplasts [11], a smaller, highly branched glycogen could help the cells achieving a higher intracellular osmotic pressure to maintain their turgor and cope more easily with osmotic stress.

5. Conclusions

The thermoacidophilic red microalga *G. sulphuraria* accumulates the most highly branched glycogen known to date. This molecule clearly differs from other glycogens in its higher percentage of branched linkages, its lack of long chains and its smaller molecular and particle size. The particular structure of this glucan could play a role in the ability of *G. sulphuraria* to adapt to nutrient-limited conditions and cope with different stresses that are characteristic from the environments that this alga colonizes.

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